Development of a yeast-based assay for bioavailable phosphorous

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ABSTRACT

Preventing eutrophication of inland freshwater ecosystems requires quantifying the phosphorous (P) content of the streams and rivers that feed them. Typical methods for measuring P assess soluble reactive P (SRP) or total P (TP) and require expensive analytical techniques that produce hazardous waste. Here we present a novel method for measuring the more relevant bioavailable P (BAP); this assay utilizes the growth of familiar baker's yeast, avoids production of hazardous waste, and reduces cost relative to measurements of SRP and TP. The yeast BAP (yBAP) assay takes advantage of the observation that yeast density at saturating growth increases linearly with provided P. We show that this relationship can be used to measure P in freshwater in concentration ranges relevant to eutrophication. In addition, we measured yBAP in water containing known amount of fertilizer and in samples from agricultural waterways. We observed that the majority of yBAP values were between those obtained from standard SRP and TP measurements, demonstrating that the assay is compatible with real-world settings. The cost-effective and nonhazardous nature of the yeast-based assay suggests that it could have utility in a range of settings, offering added insight to identify water systems at risk of eutrophication from excess phosphorus.

Keywords: eutrophication, whole cell yeast biosensor, bioavailable phosphorus.

INTRODUCTION

Eutrophication, a form of water pollution caused by excess nutrients, is a concern for inland freshwater ecosystems worldwide.[1,2] Surplus nutrient supply can lead to algal blooms, which disrupt ecosystems, often followed by algal senescence which can lead to hypoxic zones that limit biology; these impacts impact local economies by reducing commercial fishing, water recreation, and waterfront real estate.[3] In the Great Lakes region, phosphorus (P) runoff from excess fertilizer application on agricultural lands contributes significantly to recurring algal blooms.[4,5] Moreover, over application of P-based fertilizer is also costly to farmers and represents a waste of a limited resource.[6] To prevent eutrophication and reduce P waste, accurate P detection methods are needed to pinpoint where excess P is entering waterways. Standard approaches measure either the total P (TP) or soluble reactive P (SRP); SRP typically provides an artificially low estimate of BAP, while TP often provides an artificially high estimate[7]. The quantity most relevant to the biological response to eutrophication is the bioavailable P (BAP), i.e., the portion of P accessible to organisms[8].

Of the standard analyses, SRP is the easiest to perform: it relies on filtered samples in which forms of P that are both soluble and simple (i.e., orthophosphosphates) react with molybdic acid to produce a measurable color change.[9,10] While straightforward, SRP assays fail to quantify the BAP in complex molecules (e.g., DNA) or the particulate fraction (e.g., adhered to minerals in clay or present in partially decomposed organic materials). In contrast, the TP measurement captures complex or sediment-bound P forms by first subjecting the sample to high temperatures in strong acid before the

molybdic acid reaction.[11] However, through this process, TP assays can quantify P in forms that are not typically bioavailable. As such, BAP is generally expected to fall between these two standard measurements, and through a combination of biological and chemical processes (e.g., enzyme secretion, local acidification), organisms access more P than is detected by SRP[8]. However, a portion of the total P typically remains unavailable to organisms, either because it resides as large particles or because the P is in a chemical form that is biologically inert[7] .

Other non-ideal aspects of the SRP and TP assays include that they produce hazardous waste, require lab-intensive techniques, and are expensive[12]. For example, the molybdic acid reaction used in both assays requires heavy metals (e.g., stannous chloride and molybdenum), and the TP assay also requires high-temperature (e.g., boiling) acid, which is dangerous even in laboratory settings. Charges for outsourcing these assays can range from $5-$20/sample for SRP and $10-$30/sample for TP (M. Trentman, pers comm), which can be cost prohibitive for users trying to monitor their local surface water for P pollution or farmers that want to optimize their fertilizer use. Thus, there is a need for an improved P-detection method that is user-friendly and cost-effective that can accurately quantify BAP, rather than SRP or TP.

Other BAP assays do exist and can be loosely categorized as chemical, quasi-biological, and biological. Chemical methods for measuring BAP in water provide an approximation of BAP by releasing P through a combination of chemical extractions and/or enzyme treatments, and then measuring it by one of various methods such as

molybdate colorimetry (similar to SRP) or NMR.[13–16] Chemical methods for assessing BAP have some of the same drawbacks as those for measuring SRP and TP. In addition, the ability of these assays to approximate BAP likely varies with the chemical composition of the particular water sample.

The recent development of a quasi-biological method for BAP uses qPCR measurements of phosphatase gene expression in bacteria that are added to water samples,[17] but the approach requires sophisticated equipment not present in many environmental labs. Also, because the bacteria are exposed to the sample for only short periods (2-8 hours), this assay does not account for slower biological or chemical processes that allow organisms to extract P from sediments in turbid environments. As such, this method may underestimate BAP for at least some types of water bodies.

Other biological methods for detecting BAP include assays based on the growth of algae[7,8] or bacterioplankton.[18] While these approaches link BAP directly to the organisms most relevant to eutrophication, algal assays can take multiple weeks, use relatively large volumes of sample water, and require specialized growth environments.[8] Bacterioplankton-based methods can be run more rapidly, but are difficult to reproduce due to the uncharacterized nature of the cultures used thus far.[18] In summary, there is room for improved methodology in measuring BAP.

To address this problem, we designed a BAP assay based on the growth of the baker's yeast *Saccharomyces cerevisiae*; yeast are safe, easy to work with, and resistant to

hazards (e.g., viruses, pesticides) common in aquatic environments.[19,20] Additionally, yeast grow quickly and are well-adapted for lab use. The resulting yeast BAP (yBAP) assay produces a linear response at P concentrations between 4 and 40 μM P (i.e., between 31 and 1240 μg L^{-1}) and can be extended to higher concentrations (~80μM) with the use of non-linear fitting functions. We tested the yBAP assay using water samples made from fertilizer, as well as water samples from an agricultural stream, and the measured values fall between SRP and TP, indicating that the yBAP assay is compatible with real-world environmental settings. Moreover, the yBAP assay produces no hazardous waste, can be run with minimal equipment, and reduces cost relative to SRP and TP tests, suggesting that it may be a useful addition to the set of P-quantification tools used to combat eutrophication.

METHODS

Yeast Bioavailable Phosphorous (yBAP) Assays

We set out to develop a technique and methodology that uses the density to which a yeast population grows to quantify BAP environmental in water samples; as discussed below, the assay is based on the principle that the saturating level of yeast growth is linear with the concentration of a limiting nutrient.[21] We developed two variants of a yeast based method to estimate yBAP, each with a different experimental scales; one assay is conducted in 50 mL centrifuge tubes and the other in 200 μL 96 well plates (hereafter referred to as the centrifuge tube and microtiter plate assays, respectively). In both assays, the size of the yeast population that results after growing in the presence test water supplemented with all required nutrients but P is assessed by measuring the

optical density, and compared to a standard curve prepared with The centrifuge tube assay was designed to require minimal equipment, making it useful for community-based monitoring efforts and for hands-on instruction in schools. To enable low-cost parallel processing of a larger number of samples, we miniaturized our assay for use in a microtiter plate, instead of a rack of centrifuge tubes, thus increasing the testing potential for analytical labs.

Centrifuge Tube Assay: For each individual assay, we placed 5 ml of aqueous sample (unknown or a standard, as described below) in a 50 ml "Falcon" centrifuge tube containing 5ml 2X phosphate-free yeast growth media (described below), 50μM (final) ampicillin and kanamycin, and 10 mM (final) sodium citrate/citric acid at pH 4.5; antibiotics were included to discourage bacterial growth, buffers were added because yeast require an acidic environment, and the 40 ml of head space in the tube provided oxygen. Baker's yeast (*Saccharomyces cerevisiae,* described below) were inoculated into these tubes from a phosphorous-depleted stock culture to achieve a final yeast concentration of 0.05 optical density (OD) at 600 nm. We chose this OD because preliminary experiments with less yeast gave inconsistent results, perhaps because of insufficient enzyme secretion from the smaller populations. We closed the caps tightly, and incubated the tubes without shaking at 37°C for four days, at which point the culture density had plateaued. We note that we chose 37°C as an incubation temperature, instead of the typical 30°C for yeast, because 37°C incubators are more readily available. To measure the final yeast density, we mixed the cultures by inversion and measured the absorbance at 600 nm on a spectrophotometer (Genesys 10S UV-Vis,

blanked against 1x media made with double deionized (dd)H_2O).[22] When performing studies of environmental water samples, the blank (different for each sample) was prepared by using the sample in place of the deionized water to dilute the media; this process accounted for the possible presence of sediment or other particulates in the samples. We generated standard curves in parallel using known amounts of freshly diluted potassium phosphate. For precision measurements, we ran both experimental and standard samples in triplicate. We describe the curve-fitting process below and include the step-by-step protocol in the supplementary material.

Microtiter Plate Assay: We miniaturized the Centrifuge Tube assay by adapting it to 96 well flat-bottom microtiter plates. The media, antibiotics, water, controls and P standards were all used in the same ratios and to the same final concentrations as the centrifuge tube assay above but scaled to a 200 µl culture in microtiter wells. We incubated the plates without shaking at 30°C for 4 days; we used the optimal yeast growth temperature of 30°C under the assumption that labs with plate readers would likely have 30°C incubators. We took OD readings of the plate (including the blanks) at 600 nm on a Synergy HI plate reader at the University of Notre Dame's Center for Environmental Science and Technology (CEST) set to 30°C; before reading, we shook samples using an orbital formation for 1 min, at 807 cycles per minute, in order to mix and suspend the yeast in each sample well. Please see supplementary material for step-by-step protocol, and we describe curve fitting below.

Calculations: For both assays, we plotted the absorbances against the known P concentrations to generate a standard curve. We found that it was best to prepare a new standard curve with each set of measurements, though under well-controlled circumstances we found that the curves are highly reproducible. To use this dataset to determine the concentrations of unknown samples, we fit the linear portion of the curve to the equation

$$OD = m * [P] + b \qquad \text{Eq. 1}$$

where OD is the optical density of yeast after incubation, [P] is the phosphorous concentration, and m and b are constants corresponding to the line slope and Y intercept respectively. To fit the whole growth curve, we used a fractional saturation equation similar to the familiar Michaelis-Menten function of biochemistry[23]

$$OD = \frac{ODMAX * [P]}{K + [P]} \qquad \text{Eq. 2}$$

where OD is the optical density of yeast after incubation with a limiting amount of P, OD_{max} is the maximum optical density of yeast after incubation (when P is no longer the limiting nutrient), [P] is the phosphorous concentration (here analogous to the substrate concentration in an enzyme reaction), and K is the empirically-determined half-saturation constant (fitted from the data).

Yeast Methods

Yeast strain: For this work, we utilized *Saccharomyces cerevisiae* strain DBY10148 (a generous gift from David Botstein, Stanford University[21]). We chose this strain because it is prototrophic, meaning that it has metabolic capabilities similar to wild yeast.

Yeast growth media: For medium-term culture maintenance (weeks-months), we streaked frozen stock cultures of yeast (in 30% glycerol) to single colonies on YPD plates ("yeast extract peptone dextrose[24]"), incubated the plates at 30° C until colonies appeared (a few days), then stored the plates at 4°C indefinitely. For most of our other manipulations in this paper we used as a foundation phosphorous-free synthetically defined (P_0SD) media as defined previously[21], which we typically prepared as 2X stock, then diluted to 1x by addition of water (ddH_20 or test water), vitamin/mineral mix, and glucose (1% final) (Table 1). We prepared P-assay media for the yeast assays by supplementing the P_0SD media with antibiotics and citrate butter (Table 1), which we included in all assays (even those made with sterile lab DI water) to maintain consistency in growth conditions between calibration curves and sample testing. To prepare standard curves, we added appropriate amounts of potassium phosphate to this 1x media from a filter-sterilized 1M potassium phosphate solution, which we made fresh every 3 months. For detailed instructions please see the supplement.

Preparation of P-depleted Stock Culture: The P-depleted stock culture was grown by inoculating 50 ml of P-limited media (made by supplementing P_0SD with 50mM potassium phosphate) with a single colony taken from a typical YPD plate. The culture was then incubated for four days at 30°C, at which time the yeast had reached the stationary phase and were depleted of their internal P storage. These P-depleted cultures were then stored at 4° C for a maximum of two weeks.

Measurements of P in fertilizer:

Assays of yBAP in Fertilizer: To test the ability of the assay to handle complex forms of phosphorus, we compared results of both yeast assays between potassium phosphate and pelletized agricultural fertilizer (i.e., phosphorite enriched with phosphorus pentoxide; obtained from Co-Alliance in Buchanan, MI) with a stated N:P:K ratio of 9:23:30, noting that the 23% P in the ratio is in reference to the P_2O_5, which is actually 44% P;[25] this means that the fertilizer is actually 10.1% P by weight. We made stock 13.6% (w/v) fertilizer solution in double deionized water, and shook the solution, while covered, at room temperature for four days to completely dissolve the pellets. These tests were performed as described in the centrifuge tube assay (10 ml cultures) and microtiter plate assay (200 µl cultures) sections above, only replacing the potassium phosphate with fertilizer solution; we then determined the yBAP content of the fertilizer as described below (see Calculations).

Assay of SRP and TP in fertilizer: To compare our yeast-assays to an existing analytical method for dissolved P, we measured the soluble reactive phosphorus (SRP) content of the potassium phosphate and the fertilizer using 0.2 M molybdic acid, ammonium molybdate in HCl, and 0.1 M stannous chloride in HCl. We estimated SRP in a 1.5 ml assay using a 2:1:1:1 ratio between water, sample, and each reagent, added in the order listed. We took OD measurements at 665 nm after 30 - 45 minutes. To measure total P (TP) in the fertilizer, we sent a fertilizer sample to Brookside Laboratories (New Bremen, OH), where they used method AOAC 2015.18 with Inductively Coupled Plasma–Optical Emission Spectrometry determination (ICP-OES),

which corresponds to method “Alternative B” which extracts the TP from the sample by boiling with HCl, before analyzing the sample with ICP-OES[26].

Measurements of P in stream samples

Sample collection: In order to test the efficacy of the yeast assay on natural water (i.e., not synthesized in the lab), we conducted the yeast assay on samples collected from a nearby agricultural stream (Shatto Ditch Watershed, IN). These samples were collected across the hydrograph of a storm (n=45), and thus likely contain a mix of chemical constituents including organic and inorganic P fertilizers, as well as pesticides and sediments. A total of 11.4 cm of rain occurred during the event, and stream discharge peaked at 1700 L s^{-1}; we began sample collection after rainfall started and continued intermittently for a week using an ISCO Automated Water Sampler (3700; Teledyne, Lincoln, NE); we then froze samples until later analysis as described below. A full summary of these samples and the context with which they were collected is available in Trentman et al.[27]; here we highlight a subset of these data to show the efficacy of the yeast growth assay on samples collected from the natural environment.

yBAP Assay on stream samples: We used the miniaturized assays when testing the stream water samples. We created a plate containing the standard curve as described above using environmentally relevant P concentrations from potassium phosphate to compare the water sample output. We ran the water samples in triplicate as described above. To indicate the presence of growth inhibitors that might be present in

environmental water samples, we added 10 μM potassium phosphate to each set of samples as an additional control.

Comparative measurement of SRP and TP from stream samples: For each stream sample, we also measured the SRP content on filtered sample (Type A/E glass fiber filter; Pall, Ann Arbor, MI; frozen until analysis) using a Lachat Quickchem Analyzer (Hach Company, Loveland, CO) and the molybdate-blue ascorbic acid method (Hach Company, Loveland CO; Method=10-115-01-1-Q). We measured TP on unfiltered samples with an in-line UV persulfate digester also on the Lachat Quickchem Analyzer (Method=10-115-01-3-E).

Chemicals and supplies

Materials: Centrifuge Tubes (Cat# 21008-951), and disposable cuvettes (Cat# 97000-586) were purchased from VWR International (Radnor, PA). Costar 3596 (Cat# EF5353F-CS), 96-well clear flat-bottom plates were purchased from Daigger Scientific (Buffalo Grove, IL). For additional chemicals and supplies, please see the Supplementary Methods.

RESULTS AND DICUSSION

Relationship between yeast growth and concentration of potassium phosphate

Our first goal to validate the assay was to establish the relationship between P concentration and yeast growth using known concentrations of P, allowing us to use this relationship to determine the amount of P in unknown samples (Figure 1A, B). The approach is based on previous work by Saldanha et al.[21], who studied yeast growth

under conditions of limiting nutrients and showed that yeast culture growth at maximum density follows a linear relationship with BAP.

As a first step, we designed a centrifuge tube assay in which yeast were grown to saturation in P-free synthetic defined media with/without known amounts of added potassium phosphate and/or test water (Figure 1A). We expected the relationship between [P] and yeast density to be linear based on previous work,[21] which would allow us to determine yBAP via linear extrapolation (Figure 1B). We found the relationship between P and yeast OD as determined from the standard curve to be both linear and highly reproducible for P concentrations between 0 and 40μM (Figure 1C). This behavior meant that at lower concentrations (<40 μM), the relationship between P and OD could be modeled by a linear fit. However, at higher P concentrations, we began to see saturation behavior (Figure 1C, inset). This saturation occurs because at higher P concentrations, other nutrients eventually become limiting, and at higher yeast concentrations, waste by-products accumulate and inhibit growth.

To extend the useful range of the assay to P concentrations > 40μM, we fit the data to a fractional saturation equation similar to the Michaelis-Menten equation, a well-known biochemical relationship that describes enzyme saturation.[23] We observed that this equation fits the data reasonably well (Figure 1C, inset), enabling samples at concentrations above the linear range (e.g., 40-80 μM) to be interpreted by extrapolation from the fitted curve in a manner analogous to the linear extrapolation (Figure 1B).

The initial centrifuge tube-based assay requires limited equipment (an incubator and a spectrophotometer), making it accessible to a wide range of users (e.g., science classrooms). However, it also requires relatively large sample sizes, occupies a large workspace, and produces substantial plastic waste. To address these concerns, we adapted the assay to the wells of a microtiter plate, and the assay gave results very similar to the centrifuge tube assay. We observed the same linear relationship between yeast growth and 0-40 μM sodium phosphate, and again found that the fractional saturation equation fit the data at higher P concentrations (Figure 1C, note green vs. blue curves). These observations indicated that the assays worked as expected with known concentrations of sodium phosphate and thus provided a foundation for attempting to use the yeast-based assay to provide a measure of the concentration of yBAP in experimental samples.

Relationship between yBAP and input phosphorus in fertilizer

As a first test of the yeast assay to determine P in an unknown sample, we measured the BAP in a sample of P_2O_5-based agricultural fertilizer. By comparing the OD of yeast grown with fertilizer to the standard curve produced with potassium phosphate (Figure 2), we found the yBAP fraction of the fertilizer to be on average 8.5 ± 1.9% using the centrifuge tube assay, and 9.8 ± 1.2% from the microtiter plate assay (Table S1). The two methods produced data that were within one standard deviation of each other supporting the precision of the yeast-based assays.

To determine the accuracy of the yeast-based assays, we compared the yBAP values to measurements of SRP and TP in the fertilizer samples, and also the amount of P expected from the documentation provided by the supplier. On average, the SRP content of the fertilizer was 9.7 ± 3.% P, which was indistinguishable from yBAP. We also analyzed fertilizer TP, which was on average 9.5% total P, again consistent with the yBAP measurement because all of the P_2O_5 was expected to be bioavailable. Moreover, all of these measurements were consistent with the P content reported by the manufacturer which was reported as 10.1% P.

To determine the useful range of the assay, we examined its ability to return the correct value for fertilizer P at different dilutions. We show that both the linear fit and saturation curve fit to data from either the Centrifuge Tube assay or the Microtiter Plate assay enable the user to measure the yBAP in input samples in the range between 4 μM and 40 μM P (Figure 3) equating to a range of 124-1240 $\mu g\ L^{-1}$. Moreover, the saturation curve fit to these data provides more accurate measurements in the range between 40 μM and 80 μM. This range is relevant to environmental P concentrations that promote eutrophication, which are generally described as occurring at concentrations > 3.2 μM (20-100 $\mu g\ L^{-1}$TP[28]). Note that the saturation curve fit could potentially be used to measure even higher P concentrations, but we recommend avoiding trying to interpret OD data that are nearing the plateau of the saturation curve, because at higher P concentrations, the OD changes little with additional input P, creating significant uncertainty in the resulting measurement.

There was general agreement among the yBAP, SRP, and TP measurements for fertilizer but because we measured both yBAP and SRP for a series of different fertilizer dilutions, we can compare the performance of the two assays at different concentrations. Although the overall differences were small, we observed that the SRP measurement slightly overestimated fertilizer P for concentrations below 16 μM and underestimated fertilizer P for concentrations above 16 μM (Figure 3). In contrast, the yBAP measurements obtained from the fractional saturation curve fit more closely matched the expected value for fertilizer P across the range tested. We note that the yBAP measurements from the linear fit underestimated the 70 μM datapoint (Figure 3), but this is to be expected because the relationship between fertilizer P and yeast density became nonlinear above 40 μM (Figure 1B, inset).

Detection of yBAP in stream water samples

The yeast-based assays were able to produce measurements of yBAP in fertilizer that were consistent with TP and SRP measurements using standard methods. Next, we tested the utility of our yeast assay with environmental water samples that would potentially have fertilizer P as well as P from other sources. We examined yBAP using n=45 water samples collected from an agricultural stream before, during, and after a storm. These water samples were expected to contain fertilizer run-off from nearby farm fields as well as environmental P from underlying substrate and decaying biomass. Here we compare yBAP using the microtiter plate assay to SRP and TP measurements using standard methods.

We found that all stream water samples fell in the linear range of the calibration curve for the microtiter plate (Figure 4) and had BAP concentrations less than 40 μM (<1,240 μg L^{-1}). We then compared BAP to the SRP and TP measurements (Figure 4B, C) and found that yBAP concentrations were generally near but slightly higher to SRP, but somewhat lower than TP (similar results were seen with analysis of an exapanded dataset, see Trentman et al.[27]). Thus the yBAP measurements fell between the TP and SRP measurements (Figure 4), which was consistent with results from measurements made using algal BAP assays.[7] In contrast, previous bacterioplankton assays have found that BAP was often below both SRP and TP.[18] Nevertheless, observations from this study provide evidence that the yeast-based assay can be used for added insight on BAP in samples obtained from field settings.

Practical recommendations and considerations in using the assay

Two variants of the assay: The Centrifuge Tube yBAP Assay was developed to minimize the need for specialized equipment, especially if the prepared culture media is already available (see supplemental methods for materials/supplies). For larger numbers of samples or cases in which the water sample size is limited, the Microtiter Plate yBAP Assay is preferable, having the same basic supply needs, except for the addition of a plate reader instead of a spectrophotometer.

Temperature: As presented here, we grew the Centrifuge Tube yBAP Assay cultures at 37°C (a common incubator temperature used for bacterial cultures) and the Microtiter Plate cultures at 30°C (the more typical temperature for yeast), but expect that either temperature could be used for either assay.

Time: As noted in the methods, we grew the yeast cultures for four days, and we recommend that users not try to shorten the growth time for two reasons. First, the linearity between yeast density and limiting nutrient is established only for the density plateau, and the situation is more complicated if the culture density is still changing when the measurements are taken. Second, it is important to give the culture time to extract P from complex forms and/or sediments. If users do try to adjust the time or other aspects of the conditions, it is important to make sure that the culture densities have reached a plateau before taking the density readings.

Possible bacterial contamination: For the reported yBAP assays, we include a combination of antibiotics to reduce bacterial contamination. In our experience, some level of remaining bacterial contamination is common when working with field samples, but small amounts of bacteria do not impact results because bacteria also scatter light, as yeast do. However, we suggest that it is important to include the antibiotics because if a situation arises where most of the light scattering comes from bacteria, rather than yeast, variation caused by presence of bacteriophages (viruses that attack bacteria) could negatively impact the yBAP results.

Possible presence of poisons or other yeast growth inhibitors in field samples: As noted above, yeast are known to be resistant to glyphosate-based herbicides.[20] In addition, they are robust to potential problems such as pH changes as long as the initial starting pH is not basic; we ensured this through the addition of citric acid buffer to the growth media (see Supplemental Methods). While we have demonstrated that the yBAP assay is compatible with field samples from an agricultural stream (Figure 4), it is always possible that a field sample would contain a poison or other growth inhibitor. To

control for this problem, we suggest that for each field sample, users include a control sample to which 20 μM phosphate-P has been added, such that if the amended sample has an OD less than that of the 20 μM standard, then the presence of inhibitors should be suspected.

Need for shaking and/or aeration: Yeast are able to grow with very minimal oxygen, such that the yeast does not need aeration during the growth period. However, yeast do settle quickly in suspension, so for reproducible results, it is important to make sure that cultures are well-mixed before reading OD.

Turbidity: Water samples collected from the field often contain sediment or other materials that could interfere with the OD measurement, but we found that the yBAP assay is compatible with moderate amounts of natural turbidity (30-4500 NTU[27]); recall that the 45 field samples were taken during a storm event. During the spectrophotometer readings, we subtracted a turbidity blank from the water sample from the OD of the yBAP reading. We suspect that as long as the sum of the OD from the yeast and the turbidity is less than ~0.7 (the point at which most spectrophotometers start to become nonlinear, see Figure S1), low levels of turbidity will not be a problem for measuring moderate levels of yBAP. As a goal for future work, we are planning to develop modified versions of the yBAP assay to be compatible with higher field turbidity through the use of non-optical methods.

Conclusions

Here we have presented a yeast-based assay for measuring bioavailable P (yBAP) in water samples, and the range of utility is compatible with the goal of detecting P

concentrations relevant to eutrophication of freshwaters (above 3.2 μM P; 20-100 μg L^{-1} TP[28]). When we tested the yBAP assay on water samples collected from an agricultural stream, we found that BAP concentrations fell between the SRP and TP values measured for the same samples, indicating that the yBAP assay has utility and provides added insight beyond standard analytical approaches. Moreover, compared to SRP and TP analyses, the yBAP assay produces less hazardous waste and requires no hazardous laboratory manipulations (e.g., hot acid for TP). The yBAP assay also reduces costs from $5-$30 per sample using the chemical analyses to $1.26-$12 per sample using the yeast-based methods (see Supplemental Material for cost analysis). Furthermore, by quantifying BAP specifically, our assay adds insight regarding the measurement of the P form that is most relevant to eutrophication. Compared to other bio-assays for BAP, our assay can be run more quickly than those based on algae[7,8] , is easier to interpret compared to those based on bacteria,[18] and requires less sophisticated equipment than qPCR-based approaches.[17] Taken together, these attributes suggest that the yBAP assay could become a useful addition to the analytical toolbox used to address the eutrophication of freshwaters.

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References

(1) Michalak, A. M.; Anderson, E. J.; Beletsky, D.; Boland, S.; Bosch, N. S.; Bridgeman, T. B.; Chaffin, J. D.; Cho, K.; Confesor, R.; Daloğlu, I.; DePinto, J. V.; Evans, M. A.; Fahnenstiel, G. L.; He, L.; Ho, J. C.; Jenkins, L.; Johengen, T. H.; Kuo, K. C.; LaPorte, E.; Liu, X.; McWilliams, M. R.; Moore, M. R.; Posselt, D. J.; Richards, R. P.; Scavia, D.; Steiner, A. L.; Verhamme, E.; Wright, D. M.; Zagorski, M. A. Record-Setting Algal Bloom in Lake Erie Caused by Agricultural and Meteorological Trends Consistent with Expected Future Conditions. *Proc. Natl. Acad. Sci.* **2013**, *110* (16), 6448–6452. https://doi.org/10.1073/pnas.1216006110.

(2) Rabalais, N. N.; Turner, R. E. Gulf of Mexico Hypoxia: Past, Present, and Future. *Limnol. Oceanogr. Bull.* **2019**, *28* (4), 117–124. https://doi.org/10.1002/lob.10351.

(3) *Ecology of Harmful Algae: 15 Tables*; Granéli, E., Turner, J. T., Eds.; Ecological studies; Springer: Berlin, 2006.

(4) Ellison, M. E.; Brett, M. T. Particulate Phosphorus Bioavailability as a Function of Stream Flow and Land Cover. *Water Res.* **2006**, *40* (6), 1258–1268. https://doi.org/10.1016/j.watres.2006.01.016.

(5) Rowland, F. E.; Stow, C. A.; Johengen, T. H.; Burtner, A. M.; Palladino, D.; Gossiaux, D. C.; Davis, T. W.; Johnson, L. T.; Ruberg, S. Recent Patterns in Lake Erie Phosphorus and Chlorophyll a Concentrations in Response to Changing Loads. *Environ. Sci. Technol.* **2020**, *54* (2), 835–841. https://doi.org/10.1021/acs.est.9b05326.

(6) Cordell, D.; White, S. Life's Bottleneck: Sustaining the World's Phosphorus for a Food Secure Future. *Annu. Rev. Environ. Resour.* **2014**, *39* (1), 161–188. https://doi.org/10.1146/annurev-environ-010213-113300.

(7) Li, B.; Brett, M. T. The Influence of Dissolved Phosphorus Molecular Form on Recalcitrance and Bioavailability. *Environ. Pollut.* **2013**, *182*, 37–44. https://doi.org/10.1016/j.envpol.2013.06.024.

(8) Dijkstra, M. L.; Auer, M. T.; Kuczynski, A.; Lambert, R. Determination of Bioavailable Phosphorus in Water Samples Using Bioassay Methods. *MethodsX* **2020**, *7*, 100807. https://doi.org/10.1016/j.mex.2020.100807.

(9) Udnan, Y.; Mckelvie, I.; Grace, M.; Jakmunee, J.; Grudpan, K. Evaluation of On-Line Preconcentration and Flow-Injection Amperometry for Phosphate Determination in Fresh and Marine Waters. *Talanta* **2005**, *66* (2), 461–466. https://doi.org/10.1016/j.talanta.2004.12.064.

(10) Rigler, F. H. The Phosphorus Fractions and the Turnover Time of Inorganic Phosphorus in Different Types of Lakes,1. *Limnol. Oceanogr.* **1964**, *9* (4), 511–518. https://doi.org/10.4319/lo.1964.9.4.0511.

(11) *Standard Methods for the Examination of Water and Wasterwater, 23rd Edition*; American Public Health Association, American Water Works Association, Water Environment Federation, 2017.
(12) Warwick, C.; Guerreiro, A.; Soares, A. Sensing and Analysis of Soluble Phosphates in Environmental Samples: A Review. *Biosens. Bioelectron.* **2013**, *41*, 1–11. https://doi.org/10.1016/j.bios.2012.07.012.
(13) Jensen, H. S.; Thamdrup, B. Iron-Bound Phosphorus in Marine Sediments as Measured by Bicarbonate-Dithionite Extraction. *Hydrobiologia* **1993**, *253* (1), 47–59. https://doi.org/10.1007/BF00050721.
(14) Jarosch, K. A.; Doolette, A. L.; Smernik, R. J.; Tamburini, F.; Frossard, E.; Bünemann, E. K. Characterisation of Soil Organic Phosphorus in NaOH-EDTA Extracts: A Comparison of 31P NMR Spectroscopy and Enzyme Addition Assays. *Soil Biol. Biochem.* **2015**, *91*, 298–309. https://doi.org/10.1016/j.soilbio.2015.09.010.
(15) Zhu, Y.; Feng, W.; Liu, S.; He, Z.; Zhao, X.; Liu, Y.; Guo, J.; Giesy, J. P.; Wu, F. Bioavailability and Preservation of Organic Phosphorus in Lake Sediments: Insights from Enzymatic Hydrolysis and 31P Nuclear Magnetic Resonance. *Chemosphere* **2018**, *211*, 50–61. https://doi.org/10.1016/j.chemosphere.2018.07.134.
(16) Cade-Menun, B. J.; Elkin, K. R.; Liu, C. W.; Bryant, R. B.; Kleinman, P. J. A.; Moore, P. A. Characterizing the Phosphorus Forms Extracted from Soil by the Mehlich III Soil Test. *Geochem. Trans.* **2018**, *19* (1), 7. https://doi.org/10.1186/s12932-018-0052-9.
(17) Yang, Y.; Yang, J.; Zhang, X. A QPCR Method to Quantify Bioavailable Phosphorus Using Indigenous Aquatic Species. *Environ. Sci. Eur.* **2018**, *30* (1), 32. https://doi.org/10.1186/s12302-018-0163-z.
(18) Jansson, M.; Berggren, M.; Laudon, H.; Jonsson, A. Bioavailable Phosphorus in Humic Headwater Streams in Boreal Sweden. *Limnol. Oceanogr.* **2012**, *57* (4), 1161–1170. https://doi.org/10.4319/lo.2012.57.4.1161.
(19) Wickner, R. B.; Fujimura, T.; Esteban, R. Viruses and Prions of Saccharomyces Cerevisiae. In *Advances in Virus Research*; Elsevier, 2013; Vol. 86, pp 1–36. https://doi.org/10.1016/B978-0-12-394315-6.00001-5.
(20) Ravishankar, A.; Pupo, A.; Gallagher, J. E. G. Resistance Mechanisms of Saccharomyces Cerevisiae to Commercial Formulations of Glyphosate Involve DNA Damage Repair, the Cell Cycle, and the Cell Wall Structure. *G3 Genes Genomes Genet.* **2020**, *10* (6), 2043–2056. https://doi.org/10.1534/g3.120.401183.
(21) Saldanha, A. J.; Brauer, M. J.; Botstein, D. Nutritional Homeostasis in Batch and Steady-State Culture of Yeast. *Mol. Biol. Cell* **2004**, *15* (9), 4089–4104. https://doi.org/10.1091/mbc.e04-04-0306.
(22) Feldmann, H. *Yeast: Molecular and Cell Biology, 2nd Edition*, 2nd ed.; 2012.
(23) Nelson, D. L.; Cox, M. M. *Lehninger Principles of Biochemistry, 7th Edition | Macmillan Learning for Instructors*; 2017.
(24) Dymond, J. S. Saccharomyces Cerevisiae Growth Media. *Methods Enzymol.* **2013**, *533*, 191–204. https://doi.org/10.1016/B978-0-12-420067-8.00012-X.

(25) University of Massachusetts Amherst. Fertilizer Calculations for Greenhouse Crops https://ag.umass.edu/greenhouse-floriculture/fact-sheets/fertilizer-calculations-for-greenhouse-crops (accessed Dec 29, 2020).
(26) Thiex, N. J. Determination of Phosphorus and Potassium in Commercial Inorganic Fertilizers by Inductively Coupled Plasma–Optical Emission Spectrometry: Single-Laboratory Validation, First Action 2015.18. *J. AOAC Int.* **2016**, *99* (4), 914–922. https://doi.org/10.5740/jaoacint.16-0050.
(27) Trentman, M. T.; Tank, J. L.; Shepherd, H. A. M.; Marrs, A. J.; Welsh, J. R.; Goodson, H. V. Understanding Disturbance Driven Variation in Bioavailable Phosphorus Using a Novel Yeast Growth Assay in an Agricultural Stream. **in revision**.
(28) Correll, D. L. The Role of Phosphorus in the Eutrophication of Receiving Waters: A Review. *J. Environ. Qual.* **1998**, *27* (2), 261–266. https://doi.org/10.2134/jeq1998.00472425002700020004x.

Tables

Table 1. Media composition

Media	Components for 1L	Use
2X Phosphorus Free Synthetically Defined Base Media (P_0SD base)	0.2 g Calcium 0.2 g Sodium chloride 1 g Magnesium sulfate 0.2 g Potassium chloride 10 g Ammonium sulfate	This is the base media for the other two medias.
Assay Ready P_0SD Media	Fill to 1L with 2x P_0SD base 20 g/L Glucose 2 ml/L 1000x Vitamins 2 ml/L 1000x Metals 2 ml/L 1000x ampicillin 2 ml/L 1000x kanamycin 20 ml/L 1M sodium citrate 20 ml/L 1M citric acid (concentrations will be halved to 1X with the addition of sample water)	Used in the assays to provide nutrients to the yeast making P the only limiting factor in the growth
Phosphorus Limited SD Media (P_{50}SD)	Fill to 1L with 1x P_0SD base 50 µl 1M potassium phosphate 10 g Glucose 1 ml 1000x Vitamins 1 ml 1000x Metals	Used to grow stock yeast cultures for assays

Figures

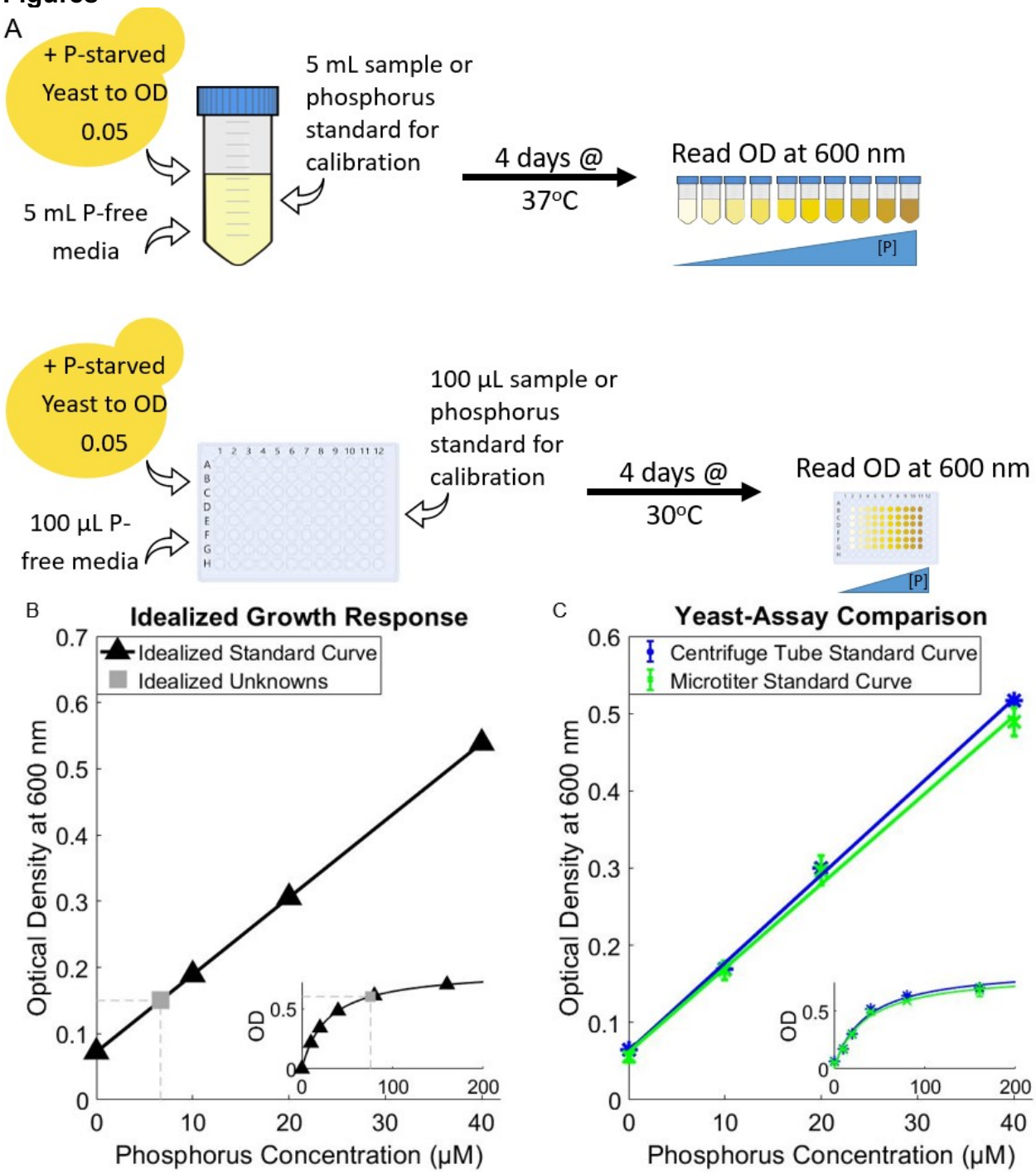

Figure 1. Comparison of an idealized growth response to the growth response found in Centrifuge Tube and Microtiter Plate Assays. (a) Centrifuge Tube assays were performed by growing P-starved yeast with media and P-containing water, either a sample or calibration standard for 4 days at 37°C. Microtiter plate assays were run for 4 days at 30°C using the same media and P-concentrations from standards or samples as

used in the Centrifuge Tube assays. The differences in experimental temperatures is due to the prevalence of 37° incubators, since our goal is for the Centrifuge Tube assay to be community friendly we felt keeping the settings as accessible to other facilities was necessary. (b) Yeast growth was expected to increase linearly with increasing amounts of phosphorus as a limiting nutrient. This would produce a function that can be used to determine the concentration of phosphorus from a given yeast sample. As an example, the grey square shows the estimated phosphorus concentration for hypothetical optical density of 0.15, using the idealized line we determine that there would be 6.65 μM phosphorus in an unknown that produced that OD. The dotted lines provide the extrapolation from optical density to the linear fit and thus the appropriate P concentration. Upon observation of the saturation effect on yeast growth, we found that a fractional saturation function could be fitted based off the Michaelis Menten equation (Nelson and Cox, 2017). Using this function a hypothetical optical density of 0.60, a sample would be determined to have a phosphorus concentration of 75.7 μM (c) Yeast growth in Centrifuge Tube Assays and Microtiter Plates. Yeast growth is linear between 0-40 μM phosphorus (linear line fitted, inset data is included to show between the entire dose response curve (Fractional saturation function fitted). The lines of the main figures represent a fitted line while the lines of the insets represent a saturation function. These functions can both be used to determine the concentration of phosphorus from a given yeast sample. As shown by the example in the idealized figure. Error bars are standard deviation; some may not be seen due to the data markers. Data represented in micromolar concentrations note: 10 μM = 0.31 ppm= 310 μg L^{-1} (a) Data gathered using 10ml yeast-based assay performed in a 50 ml Centrifuge Tubes. Data represents the average of 5 independent experiments with 3 technical replicates each. (b) Data gathered using the miniaturized yeast-based assay performed in a 96-well plate. Data represents the average of 4 independent experiments with 6 technical replicates each.

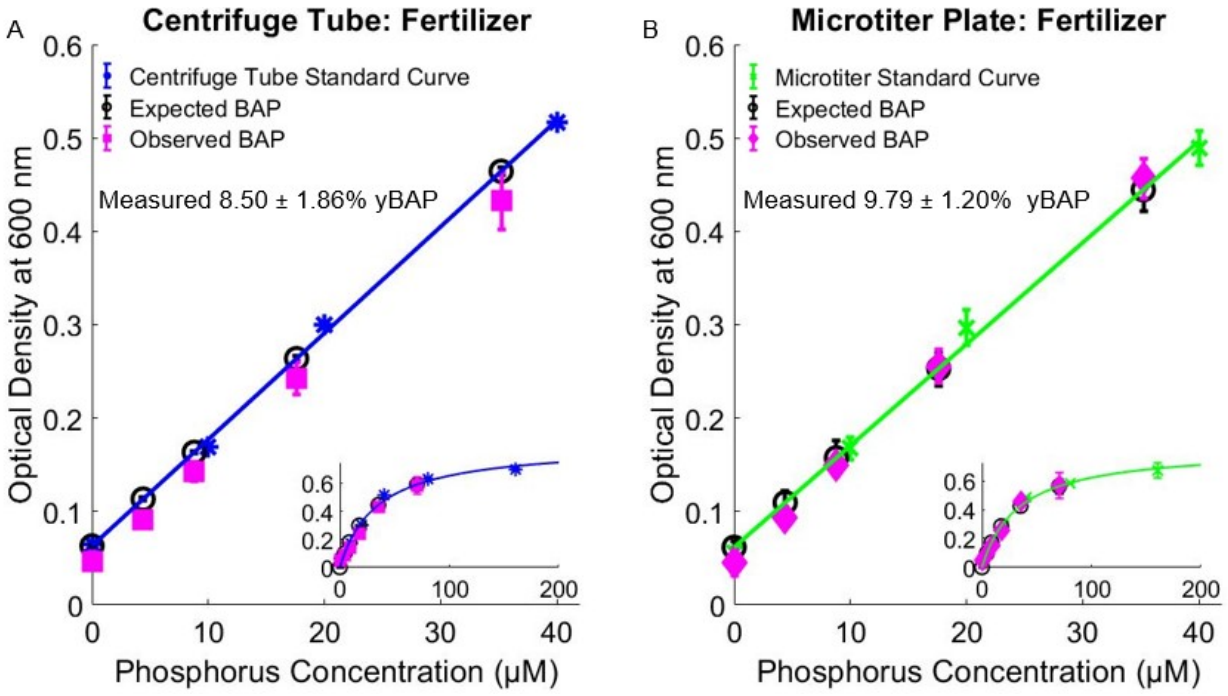

Figure 2. Use of yeast assays to detect phosphorus from fertilizer. The linear portion of the calibrations curve is shown in the main graphs (linear line fitted), the insets are the entire dose response curve (Fractional saturation function). Error bars are standard deviation; some may not be seen due to the data markers. The yeast-based assay was used to calculate the concentration of bioavailable phosphorus from the fertilizer at various dilutions, see Table 1. The concentrations of the P from the fertilizer were calculated from the 23% P_2O_5 stated by the manufacturer, which is 44% P (Center for Agriculture, Food, and the Environment, 2015). The Centrifuge Tube Assay (blue standard curve) and Microtiter Assay (green standard curve) measure the 17.6 μM P sample (40 μM P_2O_5) as 15.74 μM and 17.81 μM respectively using the linear fits and measure the 70.4 μM P sample (160 μM P_2O_5) as 68.56 μM and 66.24 μM respectively using the fractional saturation equation fits. These bioavailable phosphorus concentrations were determined by yeast growth, measured by optical density (magenta data point) and comparing these values to the values produced by the standard curves fitted with either the linear or the fractional saturation function. Using the linear portion of the curve the Centrifuge Tube data reported the fertilizer to be 8.50 ± 1.86% bioavailable phosphorus and the microtiter plate reported 9.79 ± 1.20% bioavailable phosphorus. See Table 1., for estimates from individual concentration data and Table 1S., for individual percentages. (a) Fertilizer data gathered using 10 ml yeast-based assay performed in a 50 ml Falcon (centrifuge) tubes. Data represents the average of 5 independent experiments with 3 technical replicates each. (b) Data gathered using the miniaturized yeast-based assay performed in a 96-well plate. Data represents the average of 5 independent experiments with 6 technical replicates each.

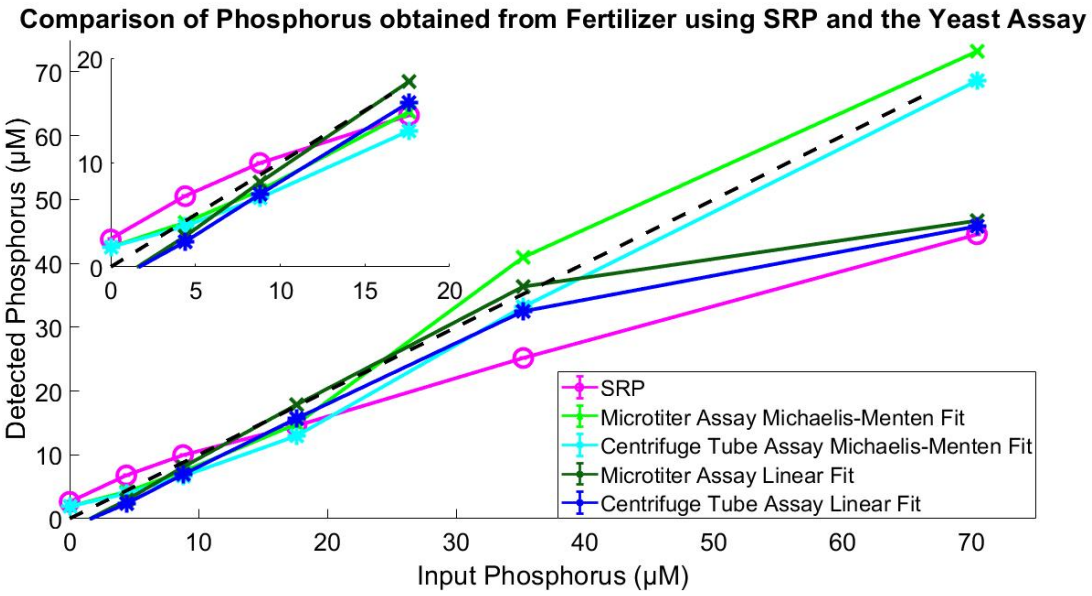

Figure 3. Comparison of fertilizer phosphorus that was detected by the SRP and the Yeast-based assay, using the standard curve in Figure 2. Lines are added to guide the eye. Error bars are standard deviation; most are obscured by the data markers. Input phosphorus is the concentration of phosphorus from the fertilizer sample (0, 4.4, 8.8, 17.6, 35.2, 70.4 μM P). The yeast-based assay was performed in a plate assay and represents the average of 5 independent experiments with 6 technical replicates each. The black dotted line represents data expected for a method that could detect all input P. The SRP detects more than input phosphorus below 16 μM and the yeast-based assay more phosphorus at higher concentrations. Above this threshold the fractional saturation fits result in higher concentrations than the total phosphorus, while the linear fits result in concentrations between the TP and SRP. See Table S1 for additional information related to this plot.

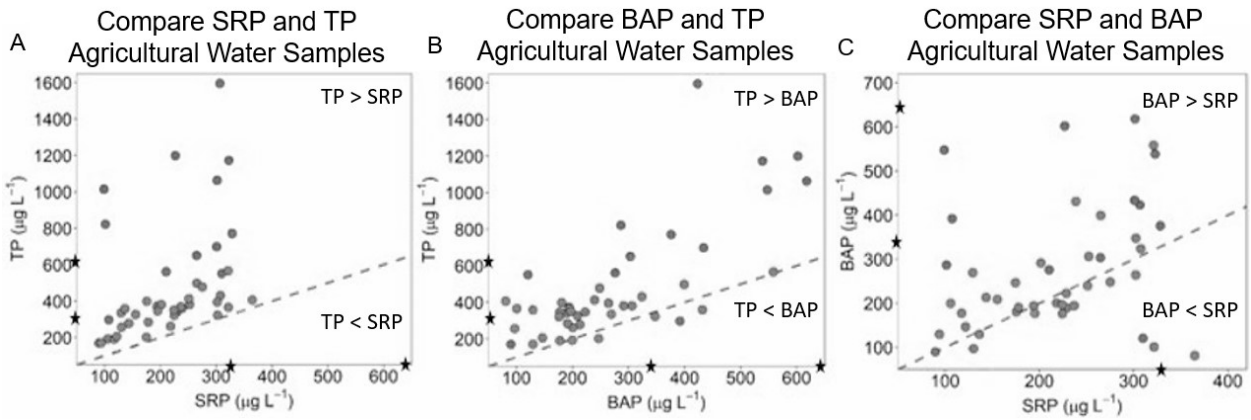

Figure 4. Water Samples. The microtiter plate assay was used to determine the concentration of BAP in agricultural water runoff samples collected in the Midwest. (a) The TP and SRP of each sample were measured using the Lachat Quickchem Analyzer, showing that the TP measurements were consistently higher than the SRP measurements. (b) The BAP values, measured using the microtiter plate assay, were consistently lower than TP. (c) The majority of the BAP values were higher than SRP values. Measurements recorded in µg L^{-1}

Supplementary Materials

Table of Contents

	Pages
1. Supplementary Tables and Figures	
1.1. Table S1	2
1.2. Table S2	2
1.3. Figure S1	3
2. Supplementary Methods	
2.1. Media component sources	4
2.2. Preparation of growth media	4-5
2.3. Preparation of phosphorus depleted yeast cultures	6
2.4. Cost Analysis	7
2.5. General notes related to running the assays	7
2.6. Centrifuge Tube Assay Protocol	8-10
2.7. Microtiter Plate Assay Protocol	11-13
3. Supplement References	14

1. Supplementary Tables and Figures

Table S1. Measurements of bioavailable phosphorus in varied dilutions of a commercial fertilizer sample as determined by linear and fractional saturation fits to the Centrifuge Tube and Microtiter Plate Assays.

	Centrifuge Tube Assay		Microtiter Assay		
Input Phosphorus based on Manufacturers Label	P from Linear Fit	P from Fractional Saturation Fit	P from Linear Fit	P from Fractional Saturation Fit	Input Phosphorus based on Brookside TP Data
4.4 μM	2.42 μM	3.93 μM	2.90 μM	4.24 μM	4.14 μM
8.8 μM	7.01 μM	6.64 μM	8.08 μM	7.35 μM	8.28 μM
17.6 μM	15.74 μM	13.03 μM	17.81 μM	14.87 μM	16.56 μM
35.2 μM	32.41 μM	33.20 μM	36.31 μM	40.94 μM	33.12 μM
70.4 μM	45.81 μM	68.56 μM	46.70 μM	73.22 μM	66.24 μM

Table S2. Calculation of the %P in a commercial fertilizer sample based on the data from varied dilutions of this fertilizer as presented in Table S1*

Input Phosphorus	Centrifuge Tube Assay						Microtiter Plate Assay					
	Linear			Fractional Saturation			Linear			Fractional Saturation		
	Average	Lower Bound	Upper Bound	Average	Lower Bound	Upper Bound	Average	Lower Bound	Upper Bound	Average	Lower Bound	Upper Bound
4.4 μM	5.55%	-0.20%	12.7%	9.04%	3.85%	11.9%	6.67%	-2.92%	20.9%	9.73%	5.37%	12.5%
8.8 μM	8.06%	4.66%	12.3%	7.64%	3.34%	9.97%	9.29%	3.53%	17.8%	8.46%	4.78%	10.7%
17.6 μM	9.05%	6.85%	11.7%	7.53%	3.50%	9.44%	10.2%	6.46%	15.8%	8.51%	5.15%	10.4%
35.2 μM	9.31%	7.74%	11.3%	9.57%	5.76%	10.8%	10.4%	7.69%	14.5%	11.8%	9.17%	12.7%
70.4 μM	6.58%	5.61%	7.8%	9.83%	9.54%	11.9%	6.71%	5.10%	9.11%	10.5%	10.0%	12.9%
Average	**7.71%**	**4.93%**	**11.2%**	**8.71%**	**5.69%**	**10.4%**	**8.67%**	**3.97%**	**15.6%**	**9.81%**	**7.47%**	**11.3%**

* To calculate these values, we determined the P from each dilution using the standard curve (see Table S1), and back calculated the dilution ratios to determine the %P in the starting material. Based on the manufacturer's label, which listed 23% P (as P_20_5), we expected 10.1% elemental P, while the TP value as reported by Brookside Labs was 9.54% P. The lower and upper bounds are 95% confidence intervals based on the lower and upper bounds of the standard curves (see calculations section in Methods).

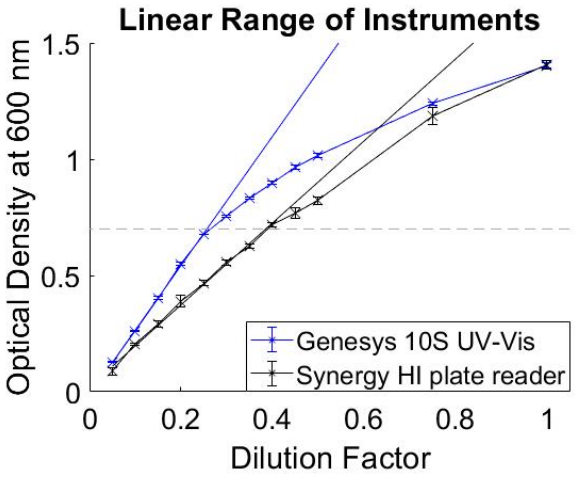

Figure S1. A single yeast culture diluted to test the linear range of each instrument. The culture was diluted to 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.75, and 1X, and the OD600 was recorded on the indicated instrument. Both the UV-Vis and the Synergy HI plate reader loose linearity near OD = 0.7, indicated by the gray dotted line. n=3 or 6 for the centrifuge tube and microtiter plate samples respectively; error bars provide the standard deviation; some are obscured by the symbols.

2. Supplementary Methods

Media Component Sources:
Phosphorus Free Media Base components: Sodium chloride (Cat# vw6430-7) and glucose (Cat# 0188) were purchased from VWR International. Potassium chloride (Cat# P217) was purchased from Fisher Scientific (Waltham, MA). Calcium chloride (Cat# 223506), magnesium chloride (Cat# 8.14733.0100), ammonium sulfate (Cat# A7523) were obtained from Sigma Aldrich (St. Louis, MO
Vitamins: Inositol (Cat# A13586), riboflavin (Cat# A11764), and thiamine hydrochloride (Cat# A19560) were purchased from Alfa Aesar (Haverhill, MA). biotin (Cat# B4501), calcium pantothenate (Cat #CS731), folic acid (Cat# F7876), Niacin (Cat #N0761), P-aminobenzoic acid (Cat #100536), and pyridoxine hydrochloride (Cat #P6280) were obtained from Sigma Aldrich .
Metals: Boric acid (Cat# BP168-1), potassium iodide (Cat# P410), and magnesium sulfate (Cat# M65) were purchased from Fisher Scientific. Copper sulfate (Cat# 209198), ferric chloride (Cat# 236489), and sodium molybdate (Cat# M1003) were purchased from Sigma Aldrich.
Antibiotics: Ampicillin (Cat# AAJ60977-06) was purchased from VWR. Kanamycin sulfate (Cat# BP906-5) was purchased by Fischer Scientific.
Other chemicals used, such as those for buffers and for the SRP test, were purchased from Sigma Aldrich.

Preparation of Phosphorus Free base Media (1X) (Saldanha *et al.,* 2004).
The media was typically prepared to a 2X stock and diluted to 1X media by test water. To prepare the 2X stock please double all ingredients below.

Fill a 1 L graduated cylinder to 500 ml with double deionized water (ddH_2O), add 0.1g calcium chloride, 0.1g sodium chloride, 0.5g magnesium chloride, 0.1 g potassium chloride and 5 g ammonium sulfate. Fill to 1 L with ddH_2O. Aliquot 500 mL into 2-1 L borosilicate glass bottles. Autoclave for 15 minutes and 15 psi at 121°C. Store at room temperature in the dark.

Glucose
A stock solution of 20 % glucose is made by dissolving 10 g of glucose powder in 50 ml of deionized water, add start stir bar before adding liquid; stir powder in slowly to avoid clumps. Filter sterilize the solution using a 0.22 µm filter, and store at room temperature in the dark. When ready to use media add 25ml of 20% glucose to 500 ml media for a final concentration of 0.95% glucose (Saldanha *et al.,* 2004).

Metals
Metals were made to 1000X concentration:
Fill a 500 mL graduated cylinder to 250 ml with ddH_2O, add 500 mg boric acid, 40 mg copper sulfate, 100mg potassium iodide, 200 mg ferric chloride, 400 mg magnesium sulfate, and 200mg sodium molybdate. Fill to 500 mL with ddH_2O. Transfer to a 1 L borosilicate glass bottle. Autoclave for 15 minutes and 15 psi at 121°C. Store at room temperature in the dark.

Vitamins
Vitamins were made to 1000X concentration:
Fill a 500 mL graduated cylinder to 250 ml with ddH_2O, add 1 mg biotin, 200 mg calcium pantothenate,1 mg folic acid, 1000mg Inositol, 200 mg Niacin, 100 mg P-aminobenzoic acid, 200 mg pyridoxine hydrochloride, 100 mg riboflavin, and 200 mg thiamine hydrochloride. Fill to 500 mL with ddH_2O. Transfer to a 1 L borosilicate glass bottle. Autoclave for 15 minutes and 15 psi at 121°C. Store at room temperature in the dark.

Once the above solutions are prepared practice sterile technique during use by using only sterile pipettes to transfer media, flaming the mouth of the bottles before and after pouring liquid and working around a flame.

Preparation of Assay-Ready Media - *typically made 50 ml at a time but can be adjusted for the number of samples being tested (need 15 ml for each control and P concentration being used to allow for triplicates)*
Combine 100 µl 1000x vitamins and metals, 100 µl 1000x ampicillin and kanamycin, 1 ml 1M sodium citrate and citric acid, and 5 ml glucose in a 50 ml graduated cylinder. Fill to 50 ml with 2X phosphorus free base media described above. Note these proportions will be appropriately diluted to 1x by addition of samples or calibration water in the assay.

Note: If members of the community would like to try our assay but are inhibited by difficulties acquiring or mixing these materials, we can provide aliquots of the metals and vitamins as well as samples of media.

Preparation of Phosphorus Limited Media
To bring phosphate levels up for growing stock cultures, phosphate limited media was made by adding glucose, vitamins, and minerals to the diluted 1X phosphate free base media as described above, and then supplementing with freshly diluted potassium phosphate to a final concentration of 50 µM. Preparation of samples for standard curves is performed in a similar way. Note that any dilutions of 1M potassium sulfate should be made fresh each day.

Phosphorus depleted yeast

Inoculate 50 ml of 1X phosphate-limited SD media with a single colony taken from a typical YPD plate and incubating for four days at 30°C, at which time they had reached the stationary phase. Allowing the yeast to reach stationary phase depletes their internal P storage so that their only P source will be from the sample or standard used in the assay.

Cost analysis of yeast assays

Microtiter plate assay

- $20/hr technician time ~1.5 hours to complete a plate
- $5.68 per microtiter plate
- ~$2 in assay ready media
- **Total: $37.68 per full plate (6 standard curve wells and 30 sample tests)**
- **$1.26 per sample**. The cost could potentially be further reduced if more samples were run per plate, e.g., by leaving out the positive controls where known [P] is added to the sample to control for the presence of possible growth inhibitors. However, unless users are confident in the absence of any possible inhibitors, we do not advise this.

Centrifuge tube assay

- $20/hr technician time ~2 hours to complete a standard set as done in our lab (24 tubes, corresponding to 6 standard curve tubes, plus six samples each with two controls)
- $0.808 per tube = $19.39
- ~$12.5 in assay ready media for a set of 24 tubes
- **Total: $71.89 for a set of 24 tubes with six samples**
- **$11.98 per sample.** As noted above, the cost could potentially be reduced if more samples were run per set, e.g., by leaving out the positive controls where known [P] is added to the sample to control for the presence of possible growth inhibitors. However, unless users are confident in the absence of any possible inhibitors, we do not advise this.

General Notes to be considered before performing either assay:

- Practice good pipetting, no bubbles.
    - The presence of bubbles in pipetting alters the true volume being added; this can result in incorrect proportions of media and samples and cause aberrant results.
- Dispose of tip after each use to avoid cross-contamination.
- If using a multichannel pipette with the microtiter plate, make sure each tip is secured and that they all intake the same volume of solution.
- Always agitate yeast mixture before aliquoting to different tubes or wells.
    - Yeast settle quickly. Proper mixing will ensure the amount of yeast delivered to each assay is the same. Failure to mix can result in aberrant results.
- Media should be stored in glassware that has been washed using phosphate-free soap and acid rinsed 3 times using 0.5 M HCl to remove any residual phosphorus.
- Contamination (e.g. from P-containing soap or remnant media) can cause considerable interference in measurements, Thus, we recommend that plastic materials be used one time only to prevent cross-contamination and residual phosphorus carry-over between experiments. If users do attempt to reuse plastic, it is essential that it be washed thoroughly.
- Laboratory yeast cultures can be poured down the drain after being sterilized by addition of bleach or alternatively by autoclaving

Centrifuge Tube Assay

Equipment needed:

• Visible light spectrometer (UV-Vis will work fine)

• 1000 µl pipetteman

• 100 or 200 µl pipetteman

Supplies needed:

• 3 – 50 ml centrifuge tubes for each sample (one for the sample and two for sample specific controls) being tested (note: 15 ml • centrifuge tubes will not work as they do not provide enough room for respiration)

• 1.5 ml plastic cuvettes for reading optical density

• 5 ml serological pipettes

• 50 ml serological pipettes

• Serological pipette bulb

• 1000 µl pipette tips

• 100 or 200 µl pipette tips

Control Samples

1. Phosphorus free media without yeast but with test water (or diluted to 1X with ddH_2O for calibration curves)
    a. This control (one for each sample) is used as a background blank, the value of which is subtracted from the OD reading for that sample. It is assembled immediately before plate reading to mitigate the possible effects of microbial growth in sample.
2. Media with yeast and 5mls of ddwater containing 20 µM P (diluted from freshly made 1mM potassium phosphate)
    a. Comparison of this control (one for each sample) to the standard curve sample with 20µM P will enable the user to determine whether possible growth inhibitors are present in the test sample.
3. Media with yeast and no phosphorus or test water (media diluted by ddH_2O instead of test water)

a. This control (one per set of tubes) will indicate the presence of phosphorus contamination in media or glassware. If the yeast remained at the starting OD, no contamination was observed. Note that this tube will also serve as the zero P point for the standard curve.

Assay Samples

1. Prepare 5 ml of assay ready media for each tube being used, but do not distribute it yet.
    a. To perform a set of 24 tubes (6 standards, and 6 samples, with two sample specific controls each) would require 120 ml of assay ready media total. However only 90 mls of media (18 tubes worth) are needed to start the assay, since the negative controls (sample blanks) will be made the day of the final reading to avoid possible interference by bacterial growth.
2. Add phosphorus depleted yeast to the prepared assay ready media; use and amount sufficient to achieve a final optical density of 0.05 at 600nm.
    a. For the 24 tubes described above, 18 require yeast (the blanks do not). Thus, for a starting yeast concentration of 0.650, you would add 6.92 ml of yeast to the 90 ml of assay ready media.
3. Prepare 5 ml of each P calibration standard in ddwater.
    a. A typical calibration curve would include 5μM, 10 μM, 20 μM, 40 μM, and 80 μM P. Note that after this 5 ml is mixed with media, the final [P] will be halved, but because the samples will be similarly diluted, this does not cause problems.
    b. P should be diluted from 1mM potassium phosphate freshly made that day from 1M potassium phosphate.
4. Aliquot 5 ml of yeast and media into each 50 ml centrifuge tube (except those being saved for the sample blanks)
5. Add 5 ml of control water, calibration standard or water sample to each tube containing media and yeast.
6. Incubate at 37°C without shaking for 4 days.
    a. Though lack of shaking and 37° are unusual for yeast, we designed the assay to be done at 37° and without shaking to minimize equipment needs
    b. We do not recommend shortening the time of incubation because the yeast may need time to extract P from complex substrates.

7. For each sample, prepare a blank by filling a tube with 5 ml of assay ready media and 5 ml of sample (plus one with ddwater). This should be done immediately before the optical density is taken.
8. Read optical density at 600 nm.
    a. For each sample, blank using the mixture of freshly diluted assay ready media and the test water mentioned above.
    b. Each sample should be mixed immediately before reading because yeast will settle to the bottom of their container if left still for any period of time.

Microtiter Plate Assay

Equipment needed:

- Plate reader with 600 nm filter
- 1000 µl pipettemen
- 100 or 200 µl pipettemen

Supplies needed:

- 1 – 96 well flat bottom plate per 10 samples (200 µl wells)
- 5 ml serological pipettes
- 50 ml serological pipettes
- Serological pipette bulb
- 1000 µl pipette tips
- 100 or 200 µl pipette tips

Control Samples

1. Phosphorus free media (diluted to 1X by test water or ddH_2O for calibration curves)
    a. This control (one for each sample) is used as a background blank , the value of which is subtracted from the OD reading for that sample. It is assembled immediately before plate reading to mitigate the possible effects of microbial growth in sample.
2. Media with yeast with 5 ml ddwater containing 20 µM P (diluted from freshly made 1mM potassium phosphate)
    a. Comparison of this control (one for each sample) to the standard curve sample with 20µM P will enable the user to determine whether possible growth inhibitors are present in the test sample.
3. Media with yeast and no phosphorus or test water (media diluted by ddH_2O instead of test water)
    a. This control (one per plate) will indicate the presence of phosphorus contamination in media or glassware. If the yeast remained at the starting OD, no contamination was observed. Note that this well will also serve as the zero P point for the standard curve.

Assay Samples:

1. Prepare 100 µl of assay ready media for each well being used, but do not distribute it yet.
    a. To perform a full plate (6 standards, and 30 samples (with two sample specific controls each)), would require 9.6 ml of assay ready media total, however only 6.6 ml of media is needed to start the assay as the negative controls (sample blanks) will be made the day of the final reading to avoid possible interference by bacterial growth prevent contamination.
2. Add phosphorus depleted yeast to the prepared assay ready media; use and amount sufficient to achieve a final optical density of 0.05 at 600nm
    a. For the plate described above, 66 wells require yeast (the blanks do not). Thus, for a starting yeast concentration of 0.650, you would add 508 µl of yeast to the 6.6 ml of assay ready media.
3. Prepare 100 µl of each P calibration standard in ddwater.
    a. A typical calibration curve would include 5µM, 10 µM, 20 µM, 40 µM, and 80 µM P. Note that after this 100 µl is mixed with media, the final [P] will be halved, but because the samples will be similarly diluted, this does not cause problems.
    b. P should be diluted from 1mM potassium phosphate freshly made that day from 1M potassium phosphate.
        i. Please note due to low volume of 1mM P needed to reach these concentrations it may be advantageous to make larger volumes for standards.
4. Aliquot 100 µl of yeast and media into microtiter plate wells (except those being saved for the sample blanks).
5. Add 100 µl of control water, calibration standard or water sample to each well containing media and yeast.
6. Incubate at 30°C static, no shaking, for 4 days.

7. For each sample, prepare a blank by filling a well with 100 µl of assay ready media and 100 µl sample (plus one with ddwater). This should be done immediately before the optical density is taken
8. Read optical density at 600 nm.
    a. For each sample, blank using the mixture of freshly diluted assay ready media and the test water mentioned above.
    b. Each sample should be mixed immediately before reading because yeast will settle to the bottom of their container if left still for any period of time.

Supplement References

Saldanha, A.J., Brauer, M.J., Botstein, D., 2004. Nutritional Homeostasis in Batch and Steady-State Culture of Yeast. MBoC 15, 4089–4104. https://doi.org/10.1091/mbc.e04-04-0306